IRW

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Kazunari TAIRA *et al.*	Group Art Unit: 1636
Application No.: 10/820,820	Examiner: Not yet assigned
Filed: April 9, 2004	
	Confirmation No.: 9492
For: EXPRESSION SYSTEM FOR TRANSCRIPTION OF FUNCTIONAL NUCLEIC ACID	

MAIL STOP PGPUB
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

REQUEST FOR CORRECTED PATENT APPLICATION PUBLICATION UNDER 37 C.F.R. § 1.221(b)

On October 7, 2004, the U.S. Patent and Trademark Office published this application as Publication Number US 2004/0198689. The published application contains material mistakes that are the fault of the Office. The Office included Applicants' Preliminary Amendment in the published application, but introduced material errors into the specification and claims. Attached are copies of the relevant pages of the Preliminary Amendment and marked-up copies of the corresponding pages of the published application containing the mistakes.

A mistake is material when it affects the public's ability to appreciate the technical disclosure of the patent application publication or determine the scope of the provisional rights that Applicants may seek to enforce upon issuance of a patent. *See* M.P.E.P. §

1130. The material mistakes occur in paragraph [0013] of the specification and in claim 8. In each case the structure of the RNA that is the claimed invention has been materially altered by the omission of three nucleotides from the sequence of the RNA variant. The alterations in the RNA sequence of paragraph [0013] and claim 8 include the omission of a "c" at what should be the third position from the 5' start of the structure; that is, the sequence should read "5' - accgu . . . " rather than "5'-acgu" in the left side of the "stem" of the RNA. The structure in paragraph [013] and claim 8 also has an omission of an "a" near the 3' end of the sequence. This omission is in the right side of the "stem" of the RNA, so that the sequence currently reads (from 5' to 3') "Aagaca" but should read "Aaagaca." Finally, the "u" at the 3' terminus has been omitted from the RNA structure in paragraph [0013] and claim 8. In total, there are three nucleotides omitted from the RNA structure (the 3rd nucleotide "c", counting from the from the 5' end; and counting from the 3' end the first nucleotide "u" and the 14th nucleotide "a"), and each omission materially alters the claimed invention.

These omissions in the structure of the RNA clearly affect the public's ability to appreciate the claims presented in the patent application publication and to determine the scope of Applicants' provisional rights. Accordingly, the mistakes are material and the Office should correct them.

Applicants also note two additional errors in the published application. The first is the inclusion of an extra period "." between the words "each" and "tRNA^{VAL}-ribozyme" in lines 13-14 of paragraph [0004]. The second error is the inclusion of an extra period "." between the words "transcribed" and "ribozymes" in line 18 of paragraph [0023]. Applicants respectfully request that these errors, which were introduced by the Office,

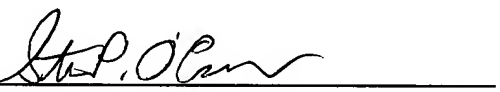
also be corrected. The corresponding pages of the application as originally filed are provided, as are marked up copies of the relevant pages of the published application.

Applicants request that the Office correct these mistakes in the published application, which are the fault of the Office, and notify Applicants of the publication of the corrected application. Applicants believe that no petition or fee is due in connection with this Request, however, if any petition or fee is due, please grant the petition and charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: December 6, 2004

By: 

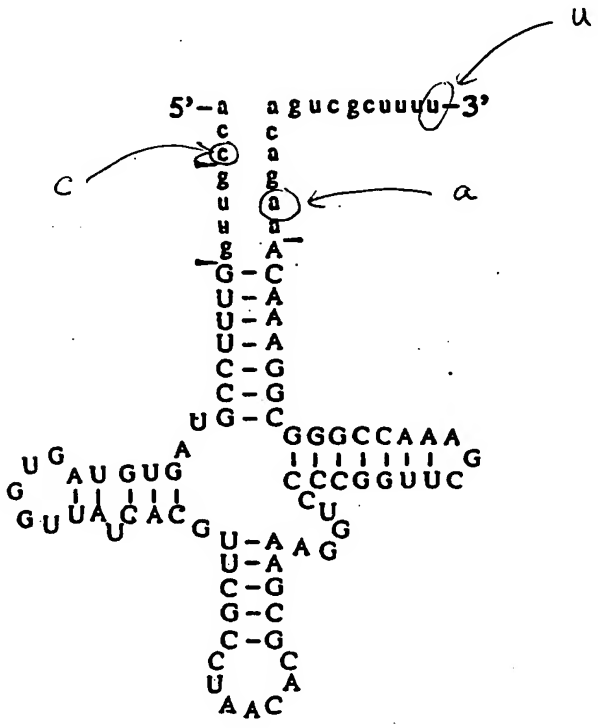
Steven P. O'Connor
Reg. No. 41,225
571-203-2718

AMENDMENTS TO THE CLAIMS:

This listing of claims will replace all prior versions and listings of claims in the application:

Claims 1-7. (Cancelled.)

8. (Currently Amended) An RNA variant adopting the following secondary structure (I), wherein said RNA variant comprises a bulge structure introduced in the region in which hydrogen bonds form between nucleotides 8 to 14 and nucleotides 73 to 79.

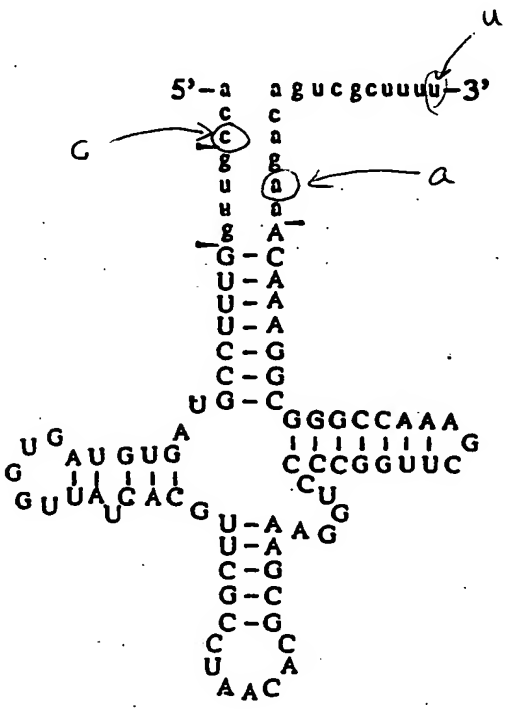


(I)

SEQ ID No. 4

9. (Original) The RNA variant according to claim 8 comprising a bulge structure which is introduced by substituting all or part of the sequence of the region of

8. An RNA variant (mature $tRNA^{Val}$) adopting the following secondary structure (I), wherein said RNA variant comprises a bulge structure introduced in the region in which hydrogen bonds form between nucleotides 8 to 14 and nucleotides 73 to 79.



(I)
(SEQ ID No. 4)

Please replace the paragraph beginning on page 14, line 19 with the following paragraph:

Figure 1 (A-E) shows the secondary structures of $tRNA^{Val}$-ribozymes that were predicted by computer folding. The sequence of hammerhead ribozyme (bold capital letters) was ligated with that of $tRNA^{Val}$ sequence (capital letters) by means of various linker sequences. The sequences that correspond to the internal promoter of seven-

$tRNA^{Val}$ is added (hereinafter termed "$tRNA^{Val}$-ribozyme")) determined not only cleavage activity but also the intracellular half-life of the ribozyme. All the chimeric $tRNA^{Val}$-ribozymes that were transcribed in the cell nucleus were exported to the cytoplasm. Thus, the ribozymes and their target were present within the same cellular compartment. Under these conditions, we found that the intracellular half-life and the steady-state level of each $tRNA^{Val}$-ribozyme were the major determinants of functional activity *in vivo*. Moreover, we demonstrated that cells that expressed a specifically designed ribozyme with the longest half-life *in vivo* were almost completely resistant to a challenge by HIV-1. Further, by establishing a small bulge structure ("bulge" refers to, in the case where RNA adopts a hairpin structure, a portion where there is a protruding single-stranded structure of unmatched base pairs) at the amino-acyl stem portion of the $tRNA^{Val}$ structure, avoidance of recognition from the mature enzyme can be achieved and as a result, any RNA sequence comprising a ribozyme sequence connected to the 3' end can be made to exist intracellularly in a form where it is connected to $tRNA^{Val}$. Any RNA comprising a ribozyme sequence connected to the 3' end of the $tRNA^{Val}$ structure of the present invention, due to the properties of the tRNA structure, is transported stably and efficiently to the cytoplasm. This is of particular importance for the intracellular function of the ribozyme.

A summary of the present invention is presented as

Also, oligonucleotide sequences necessary for the construction of these expression systems can be chemically synthesized with a DNA/RNA synthesizer (Model 394; Applied Biosystems, Division of Perkin Elmer Co. (ABI), Foster City, CA).

From predictions made using Zuker's method, it was thought that differences in the linker sequence used to connect the $tRNA^{Val}$ promoter and hammerhead ribozyme would exert great influence on the secondary structure of the recognition site of the ribozyme (See Figure 1). According to this prediction map, it was clear that whereas the overall secondary structure of the ribozyme was almost the same, the degree of freedom at the substrate-binding site differed greatly. It is clear that whereas both substrate binding sites form a stem structure within the molecule in Rz1, one binding site in Rz2, and both binding sites in Rz3 protrude to the outside. In the case of Rz3, the protruding substrate binding site may be masked by protein. However, since a ribozyme is an RNA enzyme and both binding ability and disassociation ability with a substrate are important factors in its activity, Rz3 was expected to be the best in terms of cleavage ability. We performed a reaction using intracellularly transcribed ribozymes, in an *in vitro* system under the following conditions: 40mM Tris-Cl (pH8.0), 8mM $MgCl_2$, 5mM DTT, 2mM Spermidine, 2 U/μl RNase inhibitor, 30μg total RNA. At this time, the ribozyme content in total RNA was made constant. The results showed that ribozyme activity toward

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EXPRESSION SYSTEMS FOR TRANSCRIPTION OF FUNCTIONAL NUCLIEC ACID

TECHNICAL FIELD

[0001] The present invention relates to ribozymes and their expression systems.

BACKGROUND ART

[0002] A hammerhead ribozyme is one of the smallest catalytic RNA Molecules (Kruger et al., 1982; Guerrier-Takada et al., 1983). Because of its small size and potential as an antiviral agent, numerous mechanistic studies (Dahm and Uhlenbech, 1991, Dahm et al., 1993; Eckstein and Lilley, 1996; Pontius et al., 1997; Lott et al., 1998; Zhou et al., 1996, 1997; Zhou and Taira, 1998) and studies directed towards application in vivo have been performed (Erickson and Izant, 1992; Murray, 1992; Rossi, 1995; Eckstein and Lilley, 1996; Pristei et al., 1997; Turner, 1997; Scanlon, 1997). Many successful experiments, aimed at the use of ribozymes for suppression of gene expression in different organisms, have been reported (Sarver et al., 1990; Dropulic et al., 1992; Ojwang et al, 1992; Yu et al, 1993; Zhao and Pick, 1993; Inokuchi et al, 1994; Yamada et al, 1994; Ferbeyre et al, 1996; Fujita et al, 1997; Kawasaki et al, 1998). However, the efficacy of ribozymes in vitro is not necessarily correlated with functional activity in vivo. Some of the reasons for this ineffectiveness in vivo are as follows. i) Cellular proteins may inhibit the binding of the ribozyme to its target RNA or may disrupt the active conformation of the ribozyme. ii) The intracellular concentration of metal ions essential for ribozyme-mediated cleavage might not be sufficient for functional activity. iii) Ribozymes are easily attacked by RNases. However, we are now starting to understand the parameters that determine ribozyme activity in vivo (Bertrand and Rossi, 1996; Bertrand et al., 1997; Gebhard et al., 1997). Studies in vivo have suggested that the following factors are important for the effective ribozyme-mediated inactivation of genes: a high level of ribozyme expression (Yu et al., 1993); the intracellular stability of the ribozyme (Rossi and Sarver, 1990; Eckstein and Lilley, 1996); co-localization of the ribozyme and its target RNA in the same cellular compartment (Sullenger and Cech, 1993; Bertrand et al., 1997); and the cleavage activity of the transcribed ribozyme (Thompson et al., 1995). Recently, it was shown that these various features depend on the expression system that is used (Bertrand et al., 1997).

[0003] The RNA polymerase II (pol II) system, which is employed for transcription of mRNAs, and the polymerase III (pol III) system, employed for transcription of small RNAs, such as tRNA and snRNA, have been used as ribozyme expression systems (Turner, 1997). Transcripts driven by the pol II promoter have extra sequences at the 3' and 5' ends (for example, an untranslated region, a cap structure, and a polyA tail), in addition to the coding region. These extra sequences are essential for stability in vivo and functional recognition as mRNA. A transcript containing a ribozyme sequence driven by the pol II promoter includes all those sequences, unless such sequences are trimmed after transcription (Taira et al., 1991; Ohkawa et al., 1993). As a result, in some case, the site by which the ribozyme recognizes its target may be masked, for example, by a part of the coding sequence. By contrast, the pol III system is suitable for expression of short RNAs and only very short extra sequences are generated. In addition, expression is at least one order of magnitude higher than that obtained with the pol II system (Cotten and Birnstiel, 1989). Thus, it was suggested that the pol III system might be very useful for expression of ribozymes (Yu et al., 1993; Perriman et al., 1995). However, in many cases, the expected effects of ribozymes could not be achieved in spite of the apparently desirable features of the pol III system (Ilves et al. 1996; Bertrand et al., 1997)

DISCLOSURE OF THE INVENTION

[0004] In order to investigate the parameters that determine ribozyme activity in vivo, we designed three types of ribozyme with an identical ribozyme sequence, driven by $tRNA^{Val}$ promoter which is a pol III promoter, and demonstrated that the entire structure of the transcript (ribozyme to which the sequence of $tRNA^{Val}$ is added (hereinafter termed "$tRNA^{Val}$-ribozyme")) determined not only cleavage activity but also the intracellular half-life of the ribozyme. All the chimeric $tRNA^{Val}$-ribozymes that were transcribed in the cell nucleus were exported to the cytoplasm. Thus, the ribozymes and their target were present within the same cellular compartment. Under these conditions, we found that the intracellular half-life and the steady-state level of each $tRNA^{Val}$-ribozyme were the major determinants of functional activity in vivo. Moreover, we demonstrated that cells that expressed a specifically designed ribozyme with the longest half-life in vivo were almost completely resistant to a challenge by HIV-1. Further, by establishing a small bulge structure ("bulge" refers to, in the case where RNA adopts a hairpin structure, a portion where there is a protruding single-stranded structure of unmatched base pairs) at the amino-acyl stem portion of the $tRNA^{Val}$ structure, avoidance of recognition from the mature enzyme can be achieved and as a result, any RNA sequence comprising a ribozyme sequence connected to the 3' end can be made to exist intracellularly in a form where it is connected to $tRNA^{Val}$. Any RNA comprising a ribozyme sequence connected to the 3' end of the $tRNA^{Val}$ structure of the present invention, due to the properties of the tRNA structure, is transported stably and efficiently to the cytoplasm. This is of particular importance for the intracellular function of the ribozyme.

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[0005] A summary of the present invention is presented as follows:

[0006] 1. A ribozyme comprising a nucleotide sequence having the following base sequence (I) or (II):

base sequence (I):
5'-ACCGUUGGUUUCCGUAGUGUAGUGGUUAUCACGUUCGCCUAACACGC

GAAAGGUCCCCGGUUCGAAACCGGGCACUACAAACACAACACUGAUGAGG

ACCGAAAGGUCCGAAACGGGCACGUCGGAAACCGUUUUU-3'

base sequence (II):
5'-ACCGUUGGUUUCCGUAGUGUAGUGGUUAUCACGUUCGCCUAACACGC

GAAAGGUCCCCGGUUCGAAACCGGGCACUACAAACCAACACACAACACUG

AUGAGGACCGAAAGGUCCGAAACGGGCACGUCGGAAACCGUUUUU-3'

[0007] 2. An expression vector comprising DNA encoding the ribozyme according to 1 above.

[0008] 3. A method of producing the ribozyme according to 1 above comprising transcribing to RNA with expression vector DNA as a template, wherein said expression vector DNA comprises DNA encoding the ribozyme according to 1 above.

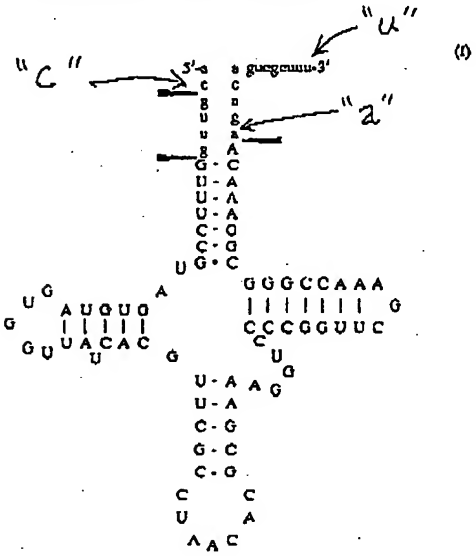
[0009] 4. A pharmaceutical composition comprising the ribozyme according to 1 above or the expression vector according to 2 above, as an effective ingredient.

[0010] 5. The pharmaceutical composition according to 4 above for the prevention and/or treatment of acquired immune deficiency syndrome.

[0011] 6. A method of specifically cleaving a target RNA using the ribozyme according to 1 above.

[0012] 7. The method of 6 above wherein the target RNA is HIV-1 RNA.

[0013] 8. An RNA variant (mature $tRNA^{Val}$) adopting the following secondary structure (I), wherein said RNA variant comprises a bulge structure introduced in the region in which hydrogen bonds form between nucleotides 8 to 14 and nucleotides 73 to 79.



[0014] 9. The RNA variant of 8 above wherein a bulge structure is introduced by substituting all or part of the sequence of the region corresponding to nucleotides 73 to 79 within a nucleotide sequence of an RNA adopting secondary structure (I).

[0015] 10. The RNA variant according to 8 above consisting of the sequence of a region corresponding to nucleotides 1-80 within a nucleotide sequence represented by SEQ ID NO: 1.

[0016] 11. The RNA variant according to 8 above consisting of the sequence of a region corresponding to nucleotides 1-86 within a nucleotide sequence represented by SEQ ID NO: 2.

[0017] 12. An RNA comprising the RNA variant of 8 above and a selected RNA chain linked thereto.

[0018] 13. The RNA according to 12 above wherein selected RNA chain is a ribozyme or an antisense RNA.

[0019] 14. The RNA according to 12 above wherein a bulge structure is formed with any nucleotide of an RNA chain linked to the 3' terminus and any nucleotide of the region of nucleotides 8 to 14 within the nucleotide sequence of an RNA adopting secondary structure (I).

[0020] 15. An expression vector comprising DNA encoding the RNA of 12 above.

[0021] Having consideration for the transcription amount, stability and post-transcription activity of ribozymes, we selected human $tRNA^{Val}$ promoter which is involved in a polymerase III system, as an expression system therefor, and examined whether there was any difference in ribozyme effect in vivo due to the way in which the ribozyme was linked to this promoter. In other words, we focussed on intracellular stability which is an important factor in obtaining significant ribozyme effect in vivo, and post-transcription activity, and set out to clarify the relationship between the high-order structure of ribozymes and these factors.

[0022] First, we designed a hammerhead ribozyme targeting a relatively conserved sequence of HIV-1, and constructed four expression systems by attaching this gene to downstream of the $tRNA^{Val}$ promoter via various sequences. As a vector for the construction of these expression systems we used pUC19 (Takara), however, other vectors such as PGREEN LANTERN (Life Technologies Oriental, Inc.) and pHaMDR (HUMAN GENE THERAPY 6:905-915 (July 1995)) may also be used. Also, oligonucleotide sequences necessary for the construction of these expression systems can be chemically synthesized with a DNA/RNA synthesizer (Model 394; Applied Biosystems, Division of Perkin Elmer Co. (ABI), Foster City, Calif.).

[0023] From predictions made using Zuker's method, it was thought that differences in the linker sequence used to connect the $tRNA^{Val}$ promoter and hammerhead ribozyme would exert great influence on the secondary structure of the recognition site of the ribozyme (See FIG. 1). According to this prediction map, it was clear that whereas the overall secondary structure of the ribozyme was almost the same, the degree of freedom at the substrate-binding site differed greatly. It is clear that whereas both substrate binding sites form a stem structure within the molecule in Rz1, one binding site in Rz2, and both binding sites in Rz3 protrude to the outside. In the case of Rz3, the protruding substrate binding site may be masked by protein. However, since a ribozyme is an RNA enzyme and both binding ability and disassociation ability with a substrate are important factors in its activity, Rz3 was expected to be the best in terms of cleavage ability. We performed a reaction using intracellularly transcribed ribozymes, in an in vitro system under the following conditions: 40 mM Tris-Cl (pH8.0), 8 mM $MgCl_2$, 5 mM DTT, 2 mM Spermidine, 2 U/μl RNase inhibitor, 30 μg total RNA. At this time, the ribozyme content in total RNA was made constant. The results showed that ribozyme activity toward short substrates that were transcribed in vitro and radioactively labeled depended on the degree of free-

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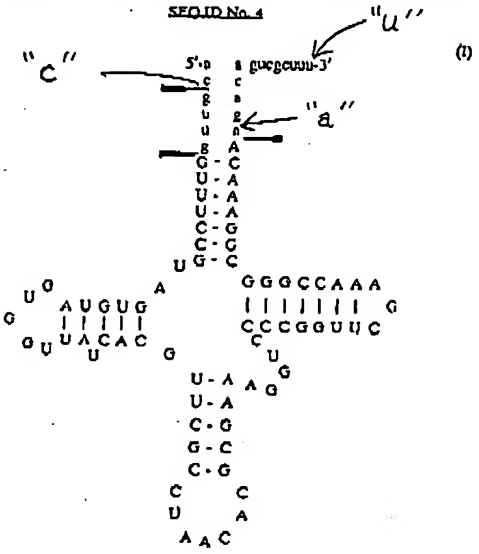
1-7. (Cancelled.)

8. An RNA variant adopting the following secondary structure (I), wherein said RNA variant comprises a bulge structure introduced in the region in which hydrogen bonds form between nucleotides 8 to 14 and nucleotides 73 to 79.

SEQ ID No. 4



9. The RNA variant according to claim 8 comprising a bulge structure which is introduced by substituting all or part of the sequence of the region of nucleotides 73 to 79 within the nucleotide sequence of an RNA adopting secondary structure (I).

10-11. (Cancelled.)

12. An RNA comprising the RNA variant according claim 8 and a selected RNA chain linked thereto.

13. The RNA according to claim 12 wherein said selected RNA chain is a ribozyme or an antisense RNA.

14. The RNA according to claim 12 wherein a bulge structure is formed with any nucleotide of an RNA chain linked to the 3' terminus and any nucleotide of the region of nucleotides 8 to 14 within the nucleotide sequence of an RNA adopting secondary structure (I).

15. An expression vector comprising DNA encoding the RNA variant of claim 8.

* * * * *